

University of Groningen

**Evidence for the Existence of a Channel in the Glucose-Specific Carrier EIIGlc of the Salmonella typhimurium Phosphoenolpyruvate-Dependent Phosphotransferase System**

Robillard, G.T.; Beechey, R.B.

*Published in:*  
Biochemistry

*DOI:*  
[10.1021/bi00354a024](https://doi.org/10.1021/bi00354a024)

**IMPORTANT NOTE:** You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
1986

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Robillard, G. T., & Beechey, R. B. (1986). Evidence for the Existence of a Channel in the Glucose-Specific Carrier EIIGlc of the Salmonella typhimurium Phosphoenolpyruvate-Dependent Phosphotransferase System. *Biochemistry*, 25(6), 1346-1354. <https://doi.org/10.1021/bi00354a024>

**Copyright**

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

**Take-down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

- Hofmann, K., Rosenthaler, J., Wells, R. D., & Yajima, H. (1964) *J. Am. Chem. Soc.* 86, 4991-4999.
- Hofmann, K., Finn, F. M., & Kiso, Y. (1978) *J. Am. Chem. Soc.* 100, 3585-3590.
- Hruby, V. J., Brewster, A. I., & Glasel, J. A. (1971) *Proc. Natl. Acad. Sci. U.S.A.* 68, 450-453.
- Jaeger, E., Thamm, P., Knof, S., Wünsch, E., Löw, M., & Kisfaludy, L. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* 359, 1617-1628.
- König, W., & Geiger, R. (1970) *Chem. Ber.* 103, 788-798.
- Lemarie, S., Yamashiro, D., Behrens, C., & Li, C. H. (1977) *J. Am. Chem. Soc.* 99, 1577-1580.
- McCormick, D. B., & Roth, J. A. (1970) *Anal. Biochem.* 34, 226-236.
- McIlhinney, J., & Schulster, D. (1974) *Endocrinology (Philadelphia)* 94, 1259-1264.
- Rae, P. A., & Schimmer, B. P. (1974) *J. Biol. Chem.* 249, 5649-5653.
- Romovacek, H., Finn, F. M., & Hofmann, K. (1983) *Biochemistry* 22, 904-909.
- Salacinski, P. R. P., McLean, C., Sykes, J. E. C., Clement-Jones, V. V., & Lowry, P. J. (1981) *Anal. Biochem.* 117, 136-146.
- Sandrin, E., & Boissonnas, R. A. (1963) *Helv. Chim. Acta* 46, 1637-1669.
- Schwyzer, R., & Kappeler, H. (1963) *Helv. Chim. Acta* 46, 1550-1572.
- Udenfriend, S., Stein, S., Böhlen, P., Dairman, W., Leimgruber, W., & Weigle, M. (1972) *Science (Washington, D.C.)* 178, 871-872.
- Yamashiro, D. (1964) *Nature (London)* 201, 76-77.

## Evidence for the Existence of a Channel in the Glucose-Specific Carrier EII<sup>Glc</sup> of the *Salmonella typhimurium* Phosphoenolpyruvate-Dependent Phosphotransferase System<sup>†</sup>

G. T. Robillard\*

Department of Physical Chemistry, University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands

R. B. Beechey

Department of Biochemistry and Agricultural Biochemistry, University College of Wales, Penglairs, Aberystwyth, SY23 3DD U.K.

Received March 15, 1985; Revised Manuscript Received October 29, 1985

**ABSTRACT:** The effect of membrane-impermeable sulfhydryl reagents on glucose-specific enzyme II (EII<sup>Glc</sup>) activity has been studied in *Salmonella typhimurium* whole cells and in properly sealed inverted cytoplasmic membrane vesicles. Glutathione *N*-hexylmaleimide and *N*-polymethylenecarboxymaleimides inactivate methyl  $\alpha$ -D-glucopyranoside ( $\alpha$ -MeGlc) transport and phosphorylation in whole cell preparations at a dithiol that can be protected by oxidizing reagents, trivalent arsenicals, or phosphorylation of EII<sup>Glc</sup>. Accessibility to this activity-linked site is restricted to small apolar reagents or to polar reagents with a hydrophobic spacer between the polar group and the reactive maleimide moiety. These same reagents inactivate  $\alpha$ -MeGlc phosphorylation in inverted cytoplasmic membrane vesicles. Inhibition results from reaction at a dithiol that can be protected by nonpermeant mercurials, oxidants, and arsenicals as well as by phosphorylation of EII. The characteristics of this site are virtually identical with those of the activity-linked dithiol elucidated in intact cells. No evidence could be found for a second activity-linked site on the other side of the membrane when the permeable reagent *N*-ethylmaleimide was used. Since only one activity-linked dithiol can be detected with sealed inverted membrane vesicles or intact cells and it is accessible to membrane-impermeable sulfhydryl reagents from both sides of the cytoplasmic membrane, we suggest that it is located in a channel constructed by the carrier and that the channel spans the membrane. A second dithiol, not essential for activity, is located near the outer surface of the cytoplasmic membrane.

The phosphoenolpyruvate-dependent methyl  $\alpha$ -D-glucopyranoside ( $\alpha$ -MeGlc)<sup>1</sup> transport activity in *Escherichia coli* and *Salmonella typhimurium* whole cells and membrane vesicles responds to two energy sources, phosphoenolpyruvate and a  $\Delta\mu_{H^+}$ . Phosphoenolpyruvate (PEP) drives  $\alpha$ -MeGlc accumulation via a P-EII<sup>Glc</sup> intermediate (Misset et al., 1983; Peri et al., 1984; Begley et al., 1982). The  $\Delta\mu_{H^+}$  inhibits  $\alpha$ -MeGlc transport (Reider et al., 1979; Hernandez-Asensio et al., 1975; Del Campo et al., 1975; Robillard & Konings,

1981). Previous studies demonstrated that phosphorylation alters the ability of sulfhydryl reagents to inactivate EII<sup>Glc</sup>

<sup>†</sup> This research was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO) and the Science and Engineering Research Council, U.K.

<sup>1</sup> Abbreviations: PEP, phosphoenolpyruvate; EII<sup>Glc</sup>, glucose-specific enzyme II of the PEP-dependent phosphotransferase system; EII<sup>Mtl</sup>, mannitol-specific enzyme II; GSM, glutathione *N*-hexylmaleimide;  $\alpha$ -MeGlc, methyl  $\alpha$ -D-glucopyranoside; DTT, dithiothreitol; AM2, -3, -5, and -10, *N*-polymethylenecarboxymaleimides containing 2, 3, 5, or 10 methylene moieties between the carboxylate group and the maleimide ring; PTS, phosphoenolpyruvate-dependent sugar phosphotransferase system; PCMBs, *p*-(chloromercuri)benzenesulfonate; DNP, 2,4-dinitrophenol; thiorin, 4-[(2-arsonophenyl)azo]-3-hydroxy-2,7-naphthalenedisulfonic acid; BAL, British antilewisite (2,3-dimercaptopropanol); Tris, tris(hydroxymethyl)aminomethane; NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

(Hagunauer-Tsapis & Kepes, 1977a,b, 1980; Robillard & Konings, 1981) and  $EII^{Mtl}$  (Roossien & Robillard, 1984). A  $\Delta\mu_{H^+}$  can also influence the reactivity of various transport proteins, including  $EII^{Glc}$ , toward sulfhydryl reagents (Robillard & Konings, 1981; Konings & Robillard, 1982; Poolman et al., 1983; Cohn et al., 1981; Le Quoc et al., 1979). The data suggested that dithiols occur in these proteins and that their oxidation states could be altered either by phosphorylation or by  $\Delta\mu_{H^+}$ . As a result of these data we proposed that turnover of a carrier might be linked to a cycling of the carrier's various redox centers at different positions across the membrane between the oxidized, disulfide, and the reduced, dithiol, state (Robillard & Konings, 1982). Our recent studies using membrane-impermeable sulfhydryl reagents have confirmed that the proline/ $H^+$  symport system in *E. coli* possesses at least two dithiol redox centers, one accessible from the outer side of the cytoplasmic membrane and the other accessible from the inner side (Poolman et al., 1983). These sites alter their redox state in response to a  $\Delta\mu_{H^+}$  or  $\Delta\Psi$  in a manner consistent with our earlier proposal.

The present investigations are intended to define the location, number, and oxidation state of the redox centers in membrane-bound  $EII^{Glc}$  as a function of the phosphorylation state of the enzyme and the energization state of the membrane.

We will examine those sites accessible from the exterior using whole cells and sites accessible from the interior using inverted cytoplasmic membranes vesicles. Both studies employ a variety of sulfhydryl-directed membrane reagents most of which are impermeable: irreversible alkylating agents, glutathione *N*-hexylmaleimide and *N*-polymethylenecarboxymaleimides; reversible mercurials, PCMBs and  $HgCl_2$ ; trivalent arsenicals, phenylarsine oxide and thorin; oxidants,  $CuCl_2$ , potassium ferricyanide, hexaamineruthenium(III) trichloride, and diamide. The differing size, charge, and polarity of these reagents offer the possibility of discriminating among various sites.

#### MATERIALS AND METHODS

**Bacterial Strain and Culture Conditions.** *S. typhimurium* PP1133 (ptsM416 trpB223) (Scholte et al., 1982) was grown on 0.1 M potassium phosphate, pH 7.2, 7.5 mM  $(NH_4)_2SO_4$ , and 0.4 mM  $MgSO_4$  containing 20 mg/L tryptophan, 0.1% yeast extract, 0.2% glucose, and, for phosphoenolpyruvate-induced cells, 0.1% PEP (monopotassium salt).

**Preparation of Whole Cells.** A culture was grown overnight at 37 °C. In the morning it was diluted 10-fold with fresh medium. When the  $OD_{660}$  reached 0.9–1.1, chloramphenicol was added to a final concentration of 50  $\mu g/mL$ , and the suspension was centrifuged. The cells were suspended in 0.1 M potassium phosphate, pH 7.3, or 0.1 M Tris-HCl, pH 8, recentrifuged, and resuspended in the same buffer. The cells were kept on ice and used within 12 h after harvesting.

**Transport Activity.**  $\alpha$ -MeGlc transport activity was measured by incubating cells with  $[^{14}C]$ - $\alpha$ -MeGlc at 30 °C. Aliquots were withdrawn, pipetted directly onto 0.45- $\mu m$  cellulose acetate filters under vacuum. The filters were washed, under vacuum, within 30 s with 3 mL of 0.1 M potassium phosphate, pH 7.3. The vacuum was released immediately thereafter, and the filters were put into scintillation vials containing 10 mL of Packard Emulsifier Scintillator 299. The vials were counted at 10 °C in a Nuclear Chicago-Mark III liquid scintillation counter after the filters became fully transparent (30–60 min). All transport and phosphorylation activities are reported as nanomoles per milligram of total protein.

**Decryptification.**  $\alpha$ -MeGlc phosphorylation activity in permeabilized cells was measured by using the toluene permeabilization procedure of Gachelin (1969).

**Preparation of Inverted Membrane Vesicles.** Inverted cytoplasmic membrane vesicles were prepared according to the procedure of Reenstra et al. (1980). The final vesicle preparation was suspended in 50 mM potassium phosphate buffer, pH 7.0, at a protein concentration of 7–10 mg/mL and stored in 50  $\mu L$  volumes in liquid nitrogen. The vesicle suspension was rapidly thawed at 40–45 °C on the day of the experiment and kept on ice until use. As a routine precaution a vesicle suspension was used only during the day that it was thawed, and the remainder was discarded at the end of the day.

**Enzyme Purification.** HPr was purified from *E. coli* P650 according to Dooijewaard et al. (1979). Enzyme I was purified from the same strain following the procedure of Robillard et al. (1979) as modified by Brouwer et al. (1982) and Misset and Robillard (1982).

**$\alpha$ -MeGlc Phosphorylation Activity.** In general a 20–40- $\mu L$  aliquot of a phosphorylating reaction mixture containing  $[^{14}C]$ - $\alpha$ -MeGlc as substrate was removed from a reaction vessel, diluted into 10 mL of water, and rapidly loaded on a Bio-Rad disposable polypropylene column containing 1 mL of Dowex AG 1-X2, 50–100 mesh, in the chloride form. The column was washed with 20 mL of distilled water to remove  $[^{14}C]$ - $\alpha$ -MeGlc. The  $[^{14}C]$ - $\alpha$ -MeGlc-P was then eluted into scintillation vials with 10 mL of 0.1 M HCl. Eight milliliters of Packard Emulsifier Scintillator 299 was added, and the samples were counted at 10 °C. The counting efficiency was 70%. Specific assay conditions are listed in the figure legends.

Protein concentration was determined according to Lowry et al. (1951).

**Reagents.** Glutathione *N*-hexylmaleimide (GSM) was prepared and isolated following the procedure of Abbot and Schachter (1976). The isolation method used by these authors involved chromatography on Sephadex G-15 in  $H_2O$ . In order to determine whether the inhibition observed in our studies could be due to the copurification of a small amount of the apolar *N,N'*-hexane-1,6-diylldimaleimide starting material, a portion of the GSM eluted from the Sephadex G-15 column was purified by using an ion-exchange procedure developed for the purification of glutathione (Furano, 1981). No difference was observed in the pattern of inhibition by the GSM preparation subjected to this extra purification step. *N*-Polymethylenecarboxymaleimides (AM2, AM3, AM5, and AM10) were synthesized as indicated by Griffiths et al. (1981).

Monobromo(trimethylammonio)bimane was purchased from Aldrich. Phosphoenolpyruvate (monopotassium salt), *p*-(chloromercuri)benzenesulfonate (PCMBs), diamide (azodicarboxylic acid bis[dimethylamide]), and phenylarsine oxide, were obtained from Sigma. Thorin [4-[2-arsonophenyl]azo]-3-hydroxy-2,7-naphthalenedisulfonic acid was purchased from Ventron Corp., Alfa Division. Hexaamineruthenium(III) trichloride was purchased from Johnson Matthey Chemicals Ltd.  $[^{14}C]$ Methyl  $\alpha$ -D-glucopyranoside was obtained from Amersham Radiochemical Centre. All other chemicals were reagent grade.

#### RESULTS

**GSM Inhibition in Cells Lacking a  $\Delta\mu_{H^+}$ .** Earlier studies demonstrated that a  $\Delta\mu_{H^+}$  alters the redox state of dithiols and their reactivity toward sulfhydryl reagents. All experiments reported in this section have been carried out on cells preincubated with a respiratory chain inhibitor, KCN (10 mM), and an uncoupler dinitrophenol (DNP, 1 mM) that abolish the  $\Delta\mu_{H^+}$  in the pH range 7–8 (data not shown). The following

protocol was employed for studying P-EII<sup>Glc</sup> or the dephosphorylated enzyme in whole cells. The cells were grown in the presence of 0.2% glucose and 0.1% PEP. They were pretreated with fluoride if we intended to examine the dephosphorylated enzyme's response to sulfhydryl reagents (Haugenauer & Kepes, 1972). After treatment with sulfhydryl reagents, PEP was added, and the PEP-dependent  $\alpha$ -MeGlc transport activity was measured (Saier et al., 1975).

Cells grown on glucose plus PEP were used for the experiments reported in Figure 1. Treatment of cells with 50 mM KF eliminates virtually all  $\alpha$ -MeGlc transport activity [Figure 1A ( $\nabla$ )]. Addition of PEP after the KF treatment ( $\Delta$ ) restores the activity to the level of that found in non-KF-treated cells ( $\diamond$ ). When the cells, depleted of PEP, are incubated with 5 mM GSM for 5 min followed by excess DTT and then loaded with PEP and assayed,  $\alpha$ -MeGlc accumulation is strongly inhibited [Figure 1A ( $\square$ )]. However, if exogenous PEP is added after the KF preincubation but before the GSM treatment, no inhibition is observed ( $\circ$ ). These data establish that GSM inactivates  $\alpha$ -MeGlc transport in intact cells and that dephosphorylated EII<sup>Glc</sup> is significantly more susceptible to inactivation than P-EII<sup>Glc</sup>.

Oxidizing agents were employed in Figure 1B to determine whether the site of GSM reaction was a monothiol or dithiol.  $\alpha$ -MeGlc accumulation is strongly inhibited in the presence of 5 mM cupric chloride [Figure 1B ( $\Delta$ )]. The inhibition can be reversed for 70% by excess DTT ( $\blacktriangle$ ). Preincubation with  $\text{Cu}^{2+}$  prior to treatment with GSM prevents irreversible inhibition by GSM ( $\bullet$ ). Diamide (5 mM), a sulfhydryl-specific oxidant, also inhibits  $\alpha$ -MeGlc accumulation reversibly ( $\diamond$ ,  $\blacklozenge$ ) and protects against GSM inhibition just as well as  $\text{Cu}^{2+}$  ( $\nabla$ ). These data are consistent with the existence of an EII<sup>Glc</sup> dithiol accessible to the nonpermeant reagents GSM and  $\text{Cu}^{2+}$  from the outer surface of the cytoplasmic membrane.

**Influence of Other Sulfhydryl Reagents.** Haugenauer and Kepes (1980) reported that, in contrast to the GSM data presented above, PCMBs was incapable of inactivating glucose transport when added to whole cells. We have repeated this experiment and confirmed their observation. Since it is possible that access to the GSM site is restricted by factors such as size, charge density, and polarity of the reagents, we have examined various reagents for their ability to inhibit  $\alpha$ -MeGlc transport and to protect against GSM inhibition.

Ferricyanide, thiorin, PCMBs, DTNB, and hexaammine-ruthenium at concentrations up to 5 mM provided no significant protection against GSM inhibition nor were they capable of inhibiting  $\alpha$ -MeGlc transport activity on their own. Phenylarsine oxide,  $\text{Cu}^{2+}$ , and diamide reversibly inhibited  $\alpha$ -MeGlc transport and protected against GSM inhibition. Slight inhibition was observed with the nonpermeant reagent monobromo(trimethylammonio)bimane whereas the *N*-polymethylenecarboxymaleimides possessing 2, 3, 5, or 10 methylene groups between the carboxylate and maleimide moieties inhibited strongly under conditions identical with those stated in the legend to Figure 1. The inhibition characteristics of AM2, the smallest and most polar of the *N*-polymethylenecarboxymaleimides, are presented in Figure 1C. At 1 mM, AM2 strongly inhibits  $\alpha$ -MeGlc accumulation ( $\nabla$ ). The inhibition is prevented when the cells are preincubated with PEP ( $\Delta$ ), 2.5 mM  $\text{CuCl}_2$  ( $\diamond$ ), or 2.5 mM diamide ( $\square$ ) prior to treatment with AM2. The patterns of inhibition and protection are identical with those observed for GSM, suggesting that both reagents are acting at the same dithiol.

**Inhibition of Transport and Phosphorylation Activity.** GSM and the AM reagents might inhibit the transport

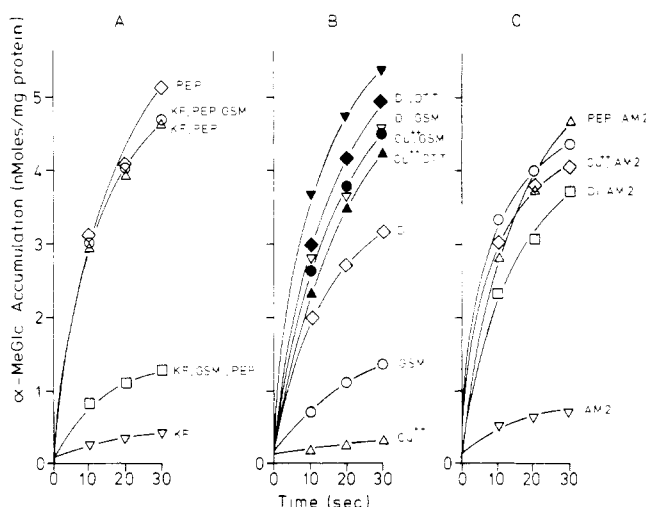


FIGURE 1: Effect of phosphorylation and oxidation on GSM and AM2 inactivation of  $\alpha$ -MeGlc transport. (A) Effect of phosphorylation on GSM inhibition. A suspension of glucose/PEP grown cells (0.22 mg of protein/mL) in 0.1 M potassium phosphate–0.4 mM  $\text{MgSO}_4$ , pH 7.3, containing 10 mM KCN and 1 mM DNP was incubated at 30 °C with the reagents and for the times listed below. Uptake measurements were initiated after the incubations by addition of [ $^{14}\text{C}$ ]- $\alpha$ -MeGlc (279 mCi/mmol) to a final concentration of between 10 and 14  $\mu\text{M}$ . Samples were withdrawn at 10, 20, and 30 s and processed as described under Materials and Methods. Incubations were carried out as follows: ( $\diamond$ ) 5 min with 0.2 M PEP; ( $\circ$ ) 5 min with 50 mM KF, 0.1 M PEP, then 5 min with 5 mM GSM, and then 2 min with 10 mM DTT; ( $\Delta$ ) 5 min with 50 mM KF plus 0.1 M PEP; ( $\square$ ) 5 min with 50 mM KF, then 5 min with 5 mM GSM, and then 2 min with 10 mM DTT plus 0.1 M PEP; ( $\nabla$ ) 5 min with 50 mM KF. (B) Effect of oxidation on inhibition by GSM. A suspension of glucose/PEP grown cells (0.22 mg of protein/mL) prepared as described in the legend to Figure 2A was incubated at 30 °C as follows: ( $\nabla$ ) 10 min with 50 mM KF and then 2 min with 10 mM DTT plus 0.1 M PEP; ( $\blacklozenge$ ) 10 min with 50 mM KF plus 5 mM diamide and then 2 min with 10 mM DTT plus 0.1 M PEP; ( $\nabla$ ) 5 min with 50 mM KF plus 5 mM diamide, then 5 min with 5 mM GSM, and then 2 min with 10 mM DTT plus 0.1 M PEP; ( $\bullet$ ) 5 min with 50 mM KF plus 5 mM  $\text{CuCl}_2$ , then 5 min with 5 mM GSM, and then 2 min with 10 mM DTT plus 0.1 M PEP; ( $\blacktriangle$ ) 10 min with 50 mM KF plus 5 mM  $\text{CuCl}_2$  and then 2 min with 10 mM DTT plus 0.1 M PEP; ( $\diamond$ ) 10 min with 50 mM KF plus 5 mM diamide, and then 2 min with 10 mM DTT plus 0.1 M PEP; ( $\Delta$ ) 10 min with 50 mM KF plus 5 mM  $\text{CuCl}_2$  and then 2 min with 0.1 M PEP (no DTT was added); ( $\circ$ ) 5 min with 50 mM KF, then 5 min with 5 mM GSM, and then 2 min with 10 mM DTT plus 0.1 M PEP. After completion of the incubation period, uptake activity was measured as stated for (A). (C) Effect of phosphorylation and oxidation on inhibition by AM2. A suspension of glucose/PEP grown cells (0.22 mg of protein/mL) prepared as described in the legend to Figure 2A was incubated at 30 °C as follows: ( $\Delta$ ) 5 min with 50 mM KF plus 0.1 M PEP, then 5 min with 1 mM AM2, and then 2 min with 10 mM DTT; ( $\circ$ ) 10 min with 50 mM KF plus 0.1 M PEP and then 2 min with 10 mM DTT; ( $\diamond$ ) 5 min with 50 mM KF plus 2.5 mM  $\text{CuCl}_2$ , then 5 min with 1 mM AM2, and then 2 min with 10 mM DTT plus 0.1 M PEP; ( $\square$ ) 5 min with 50 mM KF plus 2.5 mM diamide, then 5 min with 1 mM AM2, and then 2 min with 10 mM DTT plus 0.1 M PEP; ( $\nabla$ ) 5 min with 50 mM KF, then 5 min with 1 mM AM2, and then 2 min with 10 mM DTT plus 0.1 M PEP. Uptake activities were measured after completion of the incubation period as described for (A).

function but not the phosphorylating function of the enzyme. If so, permeabilizing the cells with toluene would allow the sugar to reach the phosphorylation site. This possibility was examined by comparing the rates of transport in GSM-treated cells with the rates of phosphorylation after the same cells had been permeabilized with toluene (Gachelin, 1969). An aliquot of the cell suspension exposed to 5 mM GSM for 10 min was permeabilized with toluene and assayed for phosphorylation activity as described under Materials and Methods. After a 10-min exposure to 4 mM GSM, 92% inhibition of the

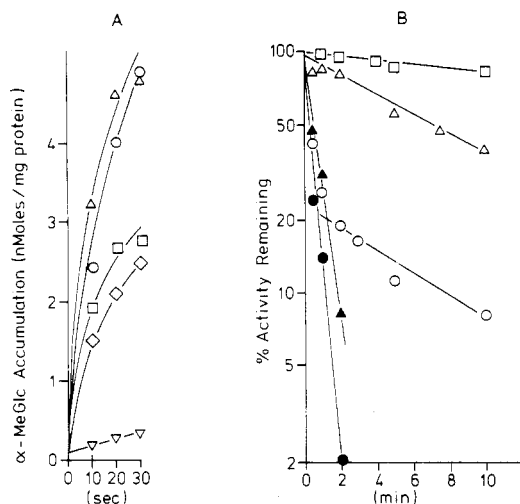


FIGURE 2: Maleimide inhibition, in the presence of PEP, of  $\alpha$ -MeGlc transport. (A) Inhibition by AM2, AM3, AM5, and AM10. A suspension of glucose/PEP grown cells (0.2 mg of protein/mL) in 0.1 M potassium phosphate–0.4 mM  $\text{MgSO}_4$  containing 10 mM KCN and 1 mM DNP was incubated at 30 °C as follows: ( $\Delta$ ) 10 min with 0.1 M PEP and then 2 min with 10 mM DTT; (O) 5 min with 0.1 M PEP, then 5 min with 3 mM AM2, and then 2 min with 10 mM DTT; ( $\diamond$ ) 5 min with 0.1 M PEP, then 5 min with 3 mM AM3, and then 2 min with 10 mM DTT; ( $\nabla$ ) 5 min with 0.1 M PEP, then 5 min with 3 mM AM10, and then 2 min with 10 mM DTT. After the incubation [ $^{14}\text{C}$ ]- $\alpha$ -MeGlc was added, and accumulation was measured as stated in the legend to Figure 1. (B) Influence of phosphorylation on the rates of inactivation by AM3 and AM10 of  $\alpha$ -MeGlc transport. Glucose/PEP grown cells were suspended to 0.2 mg of protein/mL in 0.1 M potassium phosphate–0.4 mM  $\text{MgSO}_4$ , pH 7.3. KCN was added to 10 mM, DNP to 1 mM, and KF to 50 mM. PEP was added to 100 mM if required in the incubation mixture, and the samples were incubated for 5 min at 30 °C. Two aliquots were removed for each inhibition curve and added to tubes containing DTT (final concentration 10 mM). These served as duplicate controls for each series. Inhibitors were then added. Aliquots were removed at the stated times and put into tubes containing DTT. At the end of the inhibition 100 mM PEP was added, unless it was already present, and the initial rate of  $\alpha$ -MeGlc transport was measured by adding [ $^{14}\text{C}$ ]- $\alpha$ -MeGlc to a final concentration of 28.8  $\mu\text{M}$  (specific activity 279 mCi/mMol) at  $T = 0$  and terminating the reaction at  $T = 10$  s by pipetting 50  $\mu\text{L}$  of the reaction mixture onto filters under vacuum and immediately washing the filters as described under Materials and Methods. Open symbols: inactivation in the presence of PEP. Closed symbols: inactivation in the absence of PEP. ( $\Delta$ ) Incubated with 1 mM AM3; (O) incubated with 1 mM AM10; ( $\square$ ) no maleimide added.

transport activity was observed and 90% inhibition of the phosphorylation activity occurred (data not shown). Both transport and phosphorylation of  $\alpha$ -MeGlc are inhibited by the maleimides. These data also enable us to eliminate alkylation of the PEP carrier as the reason for inhibition of  $\alpha$ -MeGlc transport. Even when PEP has free access to the PTS components, no  $\alpha$ -MeGlc phosphorylation occurs.

**Inhibition of P-EII<sup>Glc</sup>.** Figure 1 demonstrates that phosphorylation protects virtually completely against GSM and AM2 inhibition at concentrations which, in the absence of phosphorylation, inhibit  $\alpha$ -MeGlc transport for better than 90%. However, in the phosphorylated state, cells were not fully protected against inhibition by some of the other *N*-polymethylenecarboxymaleimides (Figure 2A). When cells preincubated with KCN, DNP, and PEP are exposed to 3 mM AM2 for 5 min, there is a slight inhibition (O). More substantial inhibition occurs with 3 mM AM3 ( $\diamond$ ) and 3 mM AM5 ( $\square$ ), and virtually complete inhibition occurs with 3 mM AM10 ( $\nabla$ ). The time dependence of AM3 and AM10 inhibition is presented in Figure 2B. Both reagents inactivate very rapidly at 1 mM if the cells are pretreated with 50 mM KF to elim-

inate endogenous PEP ( $\bullet$ ,  $\blacktriangle$ ). When PEP is added in a parallel experiment, the pattern of inhibition is altered. Inhibition by AM3 appears to be a monophasic, but the  $\tau_{1/2}$  for the inhibition is increased from 30 s ( $\blacktriangle$ ) to 7 min ( $\Delta$ ). Inhibition by AM10 is biphasic. The initial phase is nearly as rapid as that in the nonphosphorylated state and accounts for approximately 80% of the inhibition. This is followed by a second slower phase with a  $\tau_{1/2}$  similar to that observed with AM3. The slow inactivation is considerably more rapid than the rate at which the transport activity decreases in the absence of maleimides ( $\square$ ).

**Inhibition of P-EII<sup>Glc</sup> in the Presence of a  $\Delta\mu_{\text{H}^+}$ .** A  $\Delta\mu_{\text{H}^+}$  inhibits EII<sup>Glc</sup>-catalyzed  $\alpha$ -MeGlc phosphorylation by causing a dithiol to disulfide conversion (Robillard & Konings, 1981). The experiments presented in this section were intended to check whether the dithiol protected by oxidation in Figure 1 was also protected by a  $\Delta\mu_{\text{H}^+}$ .

Parts A and B of Figures 3 show the effect of treating PEP containing cells with GSM and the AM series alone (open symbols) or in the presence (closed symbols) of an endogenous  $\Delta\mu_{\text{H}^+}$ .  $\Delta\mu_{\text{H}^+}$  stimulates the inhibition by 3 mM AM2, AM3, and AM5 (Figure 3A), but it protects against inhibition by 3 mM AM10, and 10 mM GSM (Figure 3B). Examination of the time dependence of the inhibition process for AM3 and AM10 (Figure 3C) indicates the origin of these differences. AM10 inhibits 80% of the activity rapidly in deenergized cells pretreated with PEP (O). AM10 inhibition does not occur in energized cells ( $\bullet$ ). This is consistent with the results in Figure 3B where a 5-min preincubation with AM10 was employed. AM3 inhibits deenergized cells pretreated with PEP albeit more slowly than AM10 ( $\Delta$ ). However, energization only offers protection for the first few minutes of AM3 treatment ( $\blacktriangle$ ). Thereafter the activity decreases more rapidly than in the case of deenergized cells. This behavior is consistent with AM3 reaction at two distinct sites. During the first few minutes AM3 apparently alkylates a site that does not directly affect the activity of EII. Following reaction at the first site, the activity-linked site becomes alkylated. The higher the AM3 concentration, the shorter the period of protection and the more rapid the inactivation rate. This explains why the 5-min preincubation with 3 mM AM2, -3 and -5 resulted in more inactivation with energized than deenergized cells (Figure 3A).

The location of this new site was examined with PCMBs. Experiments reported above showed that PCMBs cannot reach the activity-linked dithiol. The data in Figure 3D shows that PCMBs is capable of protecting against reaction at the new site. When cells possessing an endogenous  $\Delta\mu_{\text{H}^+}$  were preincubated with PEP and 5 mM PCMBs, the period of complete protection against AM3 was longer and the subsequent inactivation phase was slower [Figure 3D ( $\Delta$ )] than when PCMBs was omitted [Figure 3D (O)]. When 5 mM thiorin or 5 mM  $\text{Ru}^{3+}$  was used exactly the same, protection was observed as found with PCMBs (data not shown). Since an impermeant dithiol-specific reagent and an oxidant can protect against reaction, this new site must be a dithiol near the periplasmic side of the membrane.

**Characteristics of  $\alpha$ -MeGlc Phosphorylation by Inverted Cytoplasmic Membrane Vesicles of *S. typhimurium* PP.1133.**  $\alpha$ -MeGlc phosphorylation rates measured by using inverted cytoplasmic membrane vesicles and excess  $\text{E}_1$ , HPr, and PEP yield nonlinear Lineweaver–Burk plots (Figure 4, open symbols). Detergents such as deoxycholate or octylpolyethylene glycol remove the nonlinearity largely or totally depending on the detergent concentration employed (Figure 4, filled sym-

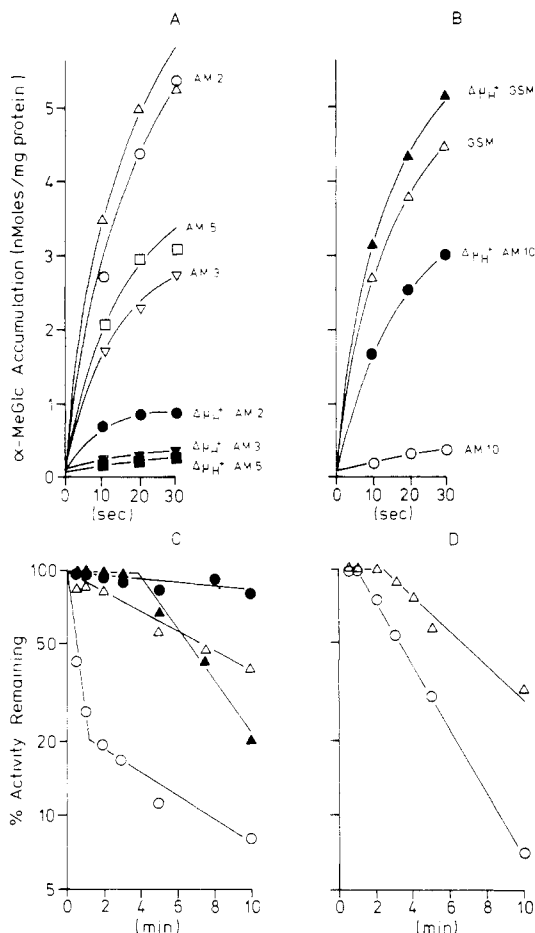


FIGURE 3: Influence of  $\Delta\mu_{H^+}$  on maleimide inhibition of  $\alpha$ -MeGlc transport. (A and B) Inhibitions in the presence of a  $\Delta\mu_{H^+}$  were done by using the following procedure. Suspension of glucose/PEP grown cells in 0.1 M potassium phosphate–0.4 mM magnesium sulfate (0.22–0.26 mg of protein/mL) were incubated for 5 min at 30 °C with 0.1 M PEP, then for 5 min with the stated maleimide, and then for 5 min with 10 mM DTT, 10 mM KCN, and 1 mM DNP.  $\alpha$ -MeGlc uptake activity was then measured as stated in the legend to Figure 1A. For inhibitions in the absence of a  $\Delta\mu_{H^+}$ , 10 mM KCN and 1 mM DNP were added along with 0.1 M PEP at the start of the incubation period. (A)  $\Delta\mu_{H^+}$  stimulation of inhibition by 3 mM AM2, AM3, and AM5. Control, no inhibitor added ( $\Delta$ ); AM2 inhibition with ( $\bullet$ ) and without ( $\circ$ )  $\Delta\mu_{H^+}$ ; AM3 inhibition with ( $\blacktriangledown$ ) and without ( $\triangledown$ )  $\Delta\mu_{H^+}$ ; AM5 inhibition with ( $\blacksquare$ ) and without ( $\square$ )  $\Delta\mu_{H^+}$ . (B)  $\Delta\mu_{H^+}$  protection against inhibition by 10 mM GSM and 3 mM AM10. 10 mM GSM inhibition with ( $\blacktriangle$ ) and without ( $\Delta$ )  $\Delta\mu_{H^+}$ ; 3 mM AM10 inhibition with ( $\bullet$ ) and without ( $\circ$ )  $\Delta\mu_{H^+}$ . (C) Rates of inhibition of  $\alpha$ -MeGlc transport activity by 1 mM AM3 and AM10 in the presence and absence of a  $\Delta\mu_{H^+}$ . The reaction mixtures were prepared as stated for (A) and (B). Aliquots were removed at the indicated times and added to the tubes containing DTT or, in the case of energized cells, DTT, KCN, and DNP (final concentrations 10, 10, and 1 mM respectively). The initial rates of  $\alpha$ -MeGlc transport were measured as stated in the legend to Figure 2B. Inhibition by AM3 ( $\Delta$ ) or AM10 ( $\circ$ ) in the presence (closed symbol) or absence (open symbols) of a  $\Delta\mu_{H^+}$ . (D) Influence of PCMBs on the rate of inactivation of  $\alpha$ -MeGlc transport activity by 1 mM AM3 in the presence of a  $\Delta\mu_{H^+}$ . The reaction mixtures were prepared as stated above for inhibitions in the presence of  $\Delta\mu_{H^+}$ . ( $\Delta$ ) Cells preincubated with 5 mM PCMBs for 5 min at 30° prior to the addition of 1 mM AM3; ( $\circ$ ) inactivation by 1 mM AM3 in the absence of PCMBs. Aliquots were removed at the indicated times and added to tubes containing 2 mM DTT, 10 mM KCN, and 1 mM DNP. The initial rates of  $\alpha$ -MeGlc transport were measured as stated in the legend to Figure 2B.

bol). Extrapolation of the linear portion of either curve results in a  $K_m$  for  $\alpha$ -MeGlc of 7–10  $\mu$ M, the expected  $K_m$  for the  $EII^{Glc}$  system. The nonlinearity in these plots is due to two populations of EII (Lolkema & Robillard, 1985). A small

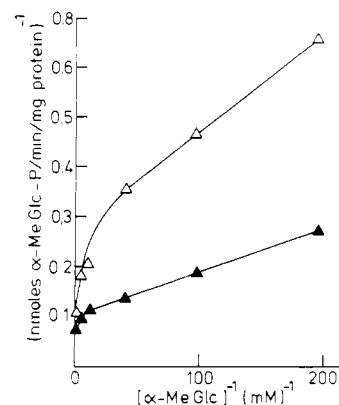


FIGURE 4: Effect of detergent on  $\alpha$ -MeGlc phosphorylation by inverted membrane vesicles. Membrane vesicles were suspended to a final protein concentration of 0.195 mg/mL in buffer containing 10 mM  $MgSO_4$ , 10 mM KF, 50 mM potassium phosphate buffer, pH 7.0, 10 mM PEP, 5 mM DTT, and 0.2% octylpoly(ethylene glycol)-300 (filled symbols) or no detergent (open symbols). Volumes of 110  $\mu$ L were incubated 5 min at 30 °C; 20  $\mu$ L of a mixture containing 70  $\mu$ M HPr, 1  $\mu$ M EI, and 100 mM PEP was then added and the 30 °C incubation continued for 5 min. Ten microliters of [ $^{14}C$ ]- $\alpha$ -MeGlc was added, and the rate of phosphorylation was determined by withdrawing four 30- $\mu$ L aliquots over a period of between 1 and 15 min and processing for [ $^{14}C$ ]- $\alpha$ -MeGlc-P.

fraction have their sugar binding site directly accessible to solution, but the major fraction have their binding sites facing the interior of the inverted membrane vesicles. Sugar must first diffuse into the vesicle before it can be phosphorylated and exported into the external medium. This diffusion process is slow and only significant at sugar concentrations above 100  $\mu$ M. The linearity at low sugar concentrations is attributable to that fraction of EII in leaky vesicles or membrane fragments whose binding sites are directly accessible to solution. Detergents cause all of the vesicles become leaky so that the entire EII population is freely accessible to the sugar. The  $V_{max}^{app}$  value<sup>2</sup> of the detergent-treated vesicles represents the total activity of the entire EII population. The  $V_{max}^{app}$  extrapolated from the linear portion of the Lineweaver–Burk plot for untreated membranes can be used to estimate the fraction of EII in leaky vesicles or fragments. The measured  $V_{max}^{app}$  for detergent-treated membranes is 14 nmol of  $\alpha$ -Glc-P min<sup>-1</sup> (mg of protein)<sup>-1</sup>. The extrapolated  $V_{max}^{app}$  for untreated membranes is 3.6 nmol min<sup>-1</sup> mg<sup>-1</sup>. Consequently 25% of the EII molecules are freely accessible to sugar in properly sealed inverted membrane vesicles. Experiments with these vesicles and nonpermeable sulfhydryl reagents can provide information concerning the accessibility of activity-linked thiols from the inner side of the cytoplasmic membrane.

**Rates of Inhibition of EII Activity by NEM and Nonpermeant Maleimides.** The rates of inactivation of EII in inverted membrane vesicles have been examined by using equal concentrations of GSM, AM2, AM5, AM10, and NEM (see Figure 5). DTT (1 mM) has been routinely added to the vesicle stock solution in the maleimide inhibition studies because we have observed that the activity-linked dithiol converts to a disulfide in the presence of oxygen alone. In this state the thiols are protected from reaction with maleimides. All reagents show similar biphasic inhibition patterns. There is a rapid inactivation with a  $T_{1/2}$  of less than 15 s. There is also

<sup>2</sup>  $EII^{Glc}$  is obligatory for the activity of this system. The vesicle preparation contains subsaturating amounts of  $EII^{Glc}$ . No extra enzyme is added for activity measurements; consequently, the y-axis intercept is only an apparent  $V_{max}$ .

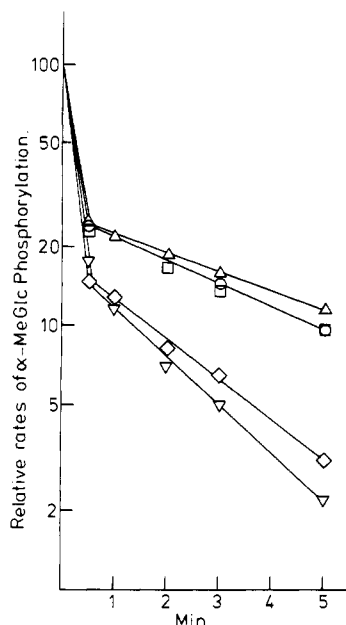


FIGURE 5: Relative rates of  $\alpha$ -MeGlc phosphorylation after exposure to maleimides for various times. Inverted membrane vesicles were suspended to a final protein concentration of 0.263 mg/mL in 10 mM  $\text{MgSO}_4$ , 10 mM KF, 50 mM potassium phosphate, pH 7.0, and 1 mM DTT. A 600  $\mu\text{L}$  volume of this mix was used per maleimide tested. The 600- $\mu\text{L}$  vesicle suspension was incubated 5 min at 30  $^\circ\text{C}$ ; 12  $\mu\text{L}$  of a 0.2 M maleimide stock solution was added followed by rapid mixing. At  $T = 0.5, 1, 2, 3$ , and 5 min, 100  $\mu\text{L}$  of the suspension was pipetted into tubes containing 5  $\mu\text{L}$  of 0.5 M DTT and mixed rapidly to neutralize the excess maleimide. A total of 5  $\mu\text{L}$  of 2% octylpoly(oxyethylene glycol)-300 and 20  $\mu\text{L}$  of a mixture containing 70  $\mu\text{M}$  HPr, 1  $\mu\text{M}$  EI, and 100 mM PEP was added to each tube, and the activity measurements were initiated by addition of 10  $\mu\text{L}$  of 3.1 mM [ $^{14}\text{C}$ ]- $\alpha$ -MeGlc (19.53 mCi/mmol). The rates of phosphorylation were determined by withdrawing 30- $\mu\text{L}$  samples at 4, 8, 12, and 16 min and processing for [ $^{14}\text{C}$ ]- $\alpha$ -MeGlc-P. The activity of the control was 10 nmol of  $\alpha$ -MeGlc-P  $\text{min}^{-1}$  (mg of protein) $^{-1}$ . (Δ) 4 mM AM5; (○) 4 mM GM; (□) 4 mM AM2; (◇) 4 mM AM10; (▽) 4 mM NEM.

a slow inactivation process with a  $T_{1/2}$  of approximately 2–5 min. Even though the patterns of inhibition are similar, the reagents seem to divide into two groups. The more hydrophobic AM10 and NEM inhibit more extensively in the first phase than do the less hydrophobic AM2, AM5, and GM. Since the inhibitions are carried out on inverted membrane vesicle suspensions prior to the addition of EI, inhibition must be attributed to the reaction of the maleimides with EII and not with EI.

**Protection against Maleimide Inhibition.** Figure 6 examines the inhibition by 3 mM AM2 and the protection against AM2 inhibition. The pattern of inhibition by 3 mM AM2 and the extent of inhibition of the unprotected enzyme (■) were virtually identical with that observed in Figure 5. The substrate,  $\alpha$ -MeGlc ( $K_m = 7 \mu\text{M}$ ), had very little if any protective effect on either phase at concentrations up to 2 mM (●). When the inverted membrane vesicles were preincubated with 4 mM PCMBs (◇) prior to the addition of 3 mM AM2, hardly any rapid or slow inhibition was observed. Since it protected, we can conclude that the rapid and slow inhibition resulted from reaction at sulfhydryl groups that were accessible from the cytoplasmic side of the membrane.

Preincubation with 2 mM thiorin (○) prior to the addition of 3 mM AM2 protected against rapid inhibition, but the slow inhibition continued with essentially the same  $T_{1/2}$  as in the unprotected sample (■). Consequently the rapid inhibition must have been at a dithiol that was accessible from the cytoplasmic side of the membrane.

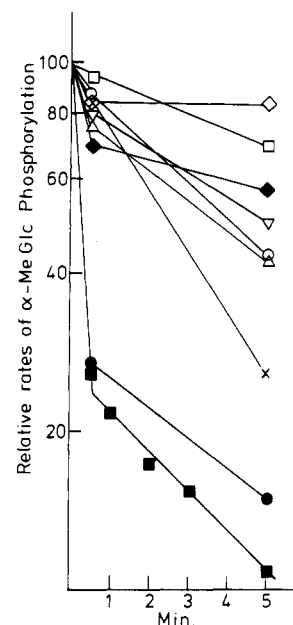


FIGURE 6: Protection against AM2 inactivation of  $\alpha$ -MeGlc phosphorylation in inverted membrane vesicles. Inverted membrane vesicles were suspended to a final protein concentration of 0.263 mg/mL in 10 mM  $\text{MgSO}_4$ , 10 mM KF, 50 mM potassium phosphate buffer, pH 7.0, and, except for (X), 1 mM DTT. The control (■) was treated following the procedure presented in the legend to Figure 4 except that 9  $\mu\text{L}$  of 0.2 M AM2 was added to a 600  $\mu\text{L}$  volume of vesicle suspension. Each series of protection experiments was executed by using the following protocol. Three tubes each containing 100  $\mu\text{L}$  of membrane vesicle suspension were incubated for 5 min at 30  $^\circ\text{C}$ . The protector was then added to each tube, and the incubation was continued for the specified time. At the end of this period 1.5  $\mu\text{L}$  of 0.2 M AM2 was added to two of the three tubes. The reaction in the first tube was terminated after 30 s by the addition of 5  $\mu\text{L}$  of 0.5 M DTT. The reaction in the second tube was terminated after 5 min by adding 5  $\mu\text{L}$  of 0.5 M DTT. The third tube served as the control for the series. It also received 5  $\mu\text{L}$  of 0.5 M DTT following the DTT addition to the second sample. A total of 5  $\mu\text{L}$  of 2% octylpoly(ethylene glycol)-300 and [except in the case of the  $\alpha$ -MeGlc protection series (●)] 20  $\mu\text{L}$  of a mixture containing 70  $\mu\text{M}$  HPr, 1  $\mu\text{M}$  EI, and 100 mM PEP was added to each tube, and the activity measurements were initiated by addition of 10  $\mu\text{L}$  of 3.1 mM [ $^{14}\text{C}$ ]- $\alpha$ -MeGlc (19.53 mCi/mmol). The rates of phosphorylation were determined as stated in the legend to Figure 5. Activity measurements for the  $\alpha$ -MeGlc protection series were initiated by the addition of the HPr/EI/PEP mix at  $T = 0$ . Protector concentration in the inactivation mixture and preincubation time: (◇) 4 mM PCMBs, 5-min preincubation; (○) 2 mM thiorin, 20-min preincubation; (▽) 1.5 mM phenylarsine oxide, 5-min preincubation; (◆) 1.5 mM  $\text{CuCl}_2$ , 5-min preincubation; (□) 10  $\mu\text{L}$  of HPr/EI/PEP mix, 2-min preincubation, and then 1.5 mM  $\text{CuCl}_2$ , 3-min preincubation; (Δ) 10  $\mu\text{L}$  of HPr/EI/PEP mix, 2-min preincubation; (●) 2.24 mM [ $^{14}\text{C}$ ]- $\alpha$ -MeGlc (3.9 mCi/mmol), 5-min preincubation; (X) influence of oxygen on AM2 inhibition. The vesicle suspension for these experiments was prepared as described at the beginning of the legend, but no DTT was added. The suspension was vortexed 2 min, and the inactivation experiments were carried out as described for the other samples.

Phenylarsine oxide (▽), a membrane-permeable arsenical, also protected only against rapid inhibition, suggesting that the slow inhibition was not due to reaction at a buried dithiol. Phosphorylation protected against GSM and AM inactivation in whole cells (Figure 1). Phosphorylation (Δ) of the inverted membrane vesicles by preincubation with PEP, HPr, and EI prior to AM2 addition also prevented the rapid inhibition but not the slow inhibition. The oxidant  $\text{CuCl}_2$  inhibits  $\alpha$ -MeGlc phosphorylation with inverted vesicles (data not shown), and it protected against AM2 inactivation [Figure 6 (◆)].  $\text{CuCl}_2$  clearly protected against both the rapid and slow inactivation; however, diamide (data not shown) and  $\text{O}_2$  [Figure 6 (X)] only



protected against the rapid inactivation. The lack of  $O_2$ , diamide, and arsenical protection against the slow inhibition indicates that the site was incapable of being oxidized or converted to a cyclic dithiol arsenite. The observed  $Cu^{2+}$  protection is most likely a result of adduct formation with the sulfhydryl group. The biphasic inactivation patterns in Figures 5 and 6 are suggestive of an equilibrium between two conformations of the carrier that interconvert very slowly or not at all. The thiols react rapidly with maleimides and can be protected by oxidants, arsenicals, or phosphorylation only in one of the conformations. Similar biphasic kinetics have been found for maleimide inactivation of  $EII^{Fru}$  from *R. sphaeroides*. Treatment of those membranes with EDTA converted the slowly reacting form into the rapidly reacting form. Zinc specifically stabilized the slowly reacting conformation (Lolkema & Robillard, 1986). EDTA and  $Zn^{2+}$  do not effect the reactivity of  $EII^{Glc}$  in an analogous way. At the present time we do not know what factors stabilize the slowly reacting form of  $EII^{Glc}$ .

**Inactivation with *N*-Ethylmaleimide.** The patterns of inactivation in whole cells with impermeant maleimides suggest that there is an  $EII$  dithiol accessible from the periplasmic side of the membrane. The data just presented on inverted vesicles argue for a dithiol with similar inactivation and protection characteristics that is accessible from the cytoplasmic side of the membrane. The following experiment attempts to determine whether there are two distinct dithiols, one accessible from each side of the membrane or only one dithiol accessible from both sides. The dithiol accessible from the cytoplasmic side can be protected by PCMBs in the inverted vesicle experiments, but the dithiol accessible from the periplasmic side in the whole cell experiments cannot be protected by PCMBs. Therefore, we have preincubated the inverted vesicles with PCMBs and then exposed them to 10 mM *N*-ethylmaleimide. In the absence of PCMBs complete inhibition is achieved by 1 mM NEM in less than 15 s. In the presence of PCMBs the cytoplasmic dithiol will be protected. If there is a separate periplasmic dithiol, it will be alkylated by the membrane-permeable NEM and result in inactivation. Virtually no inactivation occurred in the presence of PCMBs even after a 30-min incubation with 10 mM NEM. Thus, we can conclude that the dithiol accessible from the periplasmic and cytoplasmic side of the membrane is one and the same dithiol.

## DISCUSSION

Phosphorylation, imposition of a  $\Delta\mu_{H^+}$ , or exposure to oxidants protects carrier-associated dithiols from reaction with alkylating reagents (Robillard & Konings, 1981; Poolman et al., 1983; Konings & Robillard, 1982). These results plus theoretical considerations led us to propose that carrier turnover might be associated with cooperative dithiol-disulfide interchanges between different redox centers in a single carrier (Robillard & Konings, 1982). One feature of this proposal was that carriers functioning in this manner would contain at least two redox centers located at different depths in the membrane. We have provided data supporting this feature for the proline/ $H^+$  symporter from *E. coli* (Poolman et al., 1983). The present investigation addresses the same issue for the phosphoenolpyruvate-dependent phosphotransferase carriers.

The results presented in this report indicate that there are at least two operationally distinct sulfhydryl sites on  $EII^{Glc}$ . One site is a dithiol essential for  $EII^{Glc}$  phosphorylation and transport activity. The second site is a dithiol not essential for  $EII^{Glc}$  activity but capable of influencing the reactivity or accessibility of the activity-linked dithiol. These sites are

distinguishable because they react with sulfhydryl reagents under different conditions and are protected by different reagents.

**Location of the Activity-Linked Dithiol.** Combining the inactivation and protection data from all of the reagents employed in this study leads to the following view of the location of the activity-linked dithiol. It is situated in a channel or cleft of restricted dimensions no deeper than 18–20 Å from the outer surface of the cytoplasmic membrane. It is more readily accessible from the inside than from the outside of the cytoplasmic membrane. Alkylation from the outer side can only be prevented by  $Cu^{2+}$  or diamide but not by PCMBs, thiorin, ferricyanide, or hexaammineruthenium(III) trichloride. PCMBs and the smaller AM reagents are comparable in bulk and molecular weight. Since the site is accessible to the AM reagents but not to PCMBs, we suggest that access is restricted by the strongly ionized sulfate group. Both size and charge probably prevent access by thiorin, ferricyanide, and hexaammineruthenium(III) trichloride. All of the reagents, however, protect when added from the inner side (i.e., in the inverted membrane vesicle studies).

This view of the location of the activity linked dithiol assumes (i) that the distance of penetration of the maleimide group of GSM is limited by the impenetrability of the tripeptide moiety into the bulk of the membrane and also the length of the hexamethylene spacer chain and (ii) that the penetration of the acid maleimides is limited by the presence of the ionized carboxyl group and the length of the polymethylene chain. Various studies have shown that GSM and the acid maleimides are membrane impermeable at the pH, concentration, and reaction times used in the present investigations (Moore et al., 1984; Batt et al., 1976; Moore & Beechey, 1984; Poolman et al., 1983). None of the acid maleimides will inhibit 3-phosphoglyceraldehyde dehydrogenase activity in intact red blood cell ghosts (the enzyme is located on the inner face of this membrane); in contrast the corresponding *N*-alkylmaleimides have no difficulty in penetrating the membrane and inhibiting the enzyme activity. When red cell ghosts are made permeable by treatment with Triton X-100 to the AM and the substrate, there is a sharply graded ability of the acid maleimide to inhibit 3-phosphoglyceraldehyde dehydrogenase activity. The optimum chain length for inhibitory activity is found with AM2, one of the most polar of the AM series (Moore & Beechey, 1984). Consideration of the result with detergent-treated ghosts also suggests that the polymethylene chains do not fold back on themselves. If they did, the longer chain length AM would be expected to inhibit as well as AM2.

A number of reports indicate that the acid maleimides are also impermeable to mitochondrial membranes while NEM is permeable (Moore et al., 1984; Griffiths et al., 1983, 1981). Succinate dehydrogenase is accessible from the inner side of the mitochondrial membrane. When studied in submitochondrial particles, AM1, -7, and -10 were found to inhibit 50–80% of the activity at reagent concentrations of 25 nmol/mg of protein. When studied in intact mitochondria, however, no inhibition was observed with these reagents even at 50 nmol/mg of protein, yet 25 nmol of NEM resulted in complete inhibition under the same conditions (Griffiths, 1983).

Batt et al. (1976) studied the topography of the glucose carrier in erythrocytes using dextranymaleimide and glutathione maleimides. They reported that glutathione maleimide inhibits glucose efflux and that glucose protects against this inhibition. NEM also inhibits glucose efflux, but in this case,



glucose stimulates the inhibition. The authors concluded that NEM was able to reach sulfhydryl sites essential for glucose transport activity which were not accessible to GSM.

We have presented similar results indicating that GSM is incapable of penetrating the *E. coli* cytoplasmic membrane (Poolman et al., 1983). The *E. coli* proline/ $H^+$  symporter can be inhibited by GSM. The inhibition can be prevented completely by ferricyanide, thiorin, and PCMBs, suggesting that the GSM site of inhibition is a dithiol near the outer side of the cytoplasmic membrane. NEM also inhibits transport activity, but in this case, PCMBs offers only slight protection. These data indicate that NEM, an apolar reagent, is able to gain access to sites not accessible to the impermeable PCMBs. Since GSM only reacted at the PCMBs site, we can conclude that GSM has permeability characteristics similar to those of PCMBs.

**Number of Activity-Linked Dithiols.** The GSM and AM inactivation data could be indicative of two activity-linked sites situated on opposite sides of the membrane or one activity-linked site accessible from either side of the membrane. We favor a single activity-linked site for the following reasons. (i) The protection characteristics are very similar; phosphorylation protects against inactivation in whole cells and in inverted vesicles. It is difficult to envision that phosphorylating a position near the inner surface of the cytoplasmic membrane provides equivalent protection to two sites located on opposite sides of the membrane irrespective of the mechanism of protection.

(ii) NEM does not inactivate by reacting at a second site in inverted vesicles (see Results). NEM inactivates transport in whole cells just as AM2 and GSM (data not shown); therefore, it does react at the site accessible from the exterior. NEM (0.1 mM) penetrates red blood cell ghosts and inhibits 95% of the glyceraldehyde-3-P dehydrogenase within 5 min. It is safe to conclude, therefore, that the conditions used in the present study (10 mM NEM, 30-min incubation) were sufficient to have allowed penetration of the inverted vesicles and reaction at the second site, if it existed.

**Nonactivity-Linked Dithiol.** A second dithiol appears to be located nearer to the outer surface of the cytoplasmic membrane. Reaction at this site does not lead directly to inactivation; however, it alters the reactivity or accessibility of the activity-linked dithiol. The biphasic nature of the inactivation time dependence with AM2, -3, and -5 shows that the activity-linked site is protected by a  $\Delta\mu_{H^+}$  but that the protection is lost upon reaction at a second site. Since a  $\Delta\mu_{H^+}$  protects against GSM and AM10 inactivation and since the AM10 inactivation under these circumstances is monophasic, both reagents probably penetrate too deeply into the membrane to be able to react at the outer site.

**Redox State of the Dithiols.** The activity-linked dithiol is in the reduced state in the absence of a  $\Delta\mu_{H^+}$ . The initial complete protection of the phosphorylated enzyme in the presence of a  $\Delta\mu_{H^+}$  against all the maleimides tested suggests that this site might be oxidized when the membrane is energized. This would be in agreement with previous observations on this carrier (Robillard & Konings, 1981) and the proline/ $H^+$  symport system in *E. coli* (Poolman et al., 1983). Possible mechanisms for altering the redox state with a  $\Delta\mu_{H^+}$  have been explained (Robillard & Konings, 1982). It should be noted, however, that protection could also result from phosphorylation initiated changes in the reactivity of pK of the activity-linked dithiol.

No statement can be made concerning the redox state of the dithiol near the outer surface because reaction at this site

can only be detected indirectly by its effect on the reactivity of the activity-linked site. Since the outer site does influence the activity-linked site, it is worthwhile considering that the two sites may respond to each others redox state.

**Comparison of Sulfhydryl Reactivities in  $EII^{Glc}$  vs.  $EII^{Mtl}$ .**  $EII^{Glc}$  and  $EII^{Mtl}$  are both components of the phosphotransferase system, but the sequence of reactions in which they participate is not identical. The phosphoryl donor for  $EII^{Mtl}$  is P-HPr, while for  $EII^{Glc}$ , it is P- $EIII^{Glc}$ . Furthermore, the molecular mass of the two proteins differs significantly.  $EII^{Mtl}$  is approximately 60 000 daltons and  $EII^{Glc}$  is approximately 45 000 daltons. The amino acid sequence of  $EII^{Mtl}$  has been determined from the nucleotide sequence of the cloned gene (Lee & Saier, 1983). There are four cysteines in the protein. We have been able to label three of them in the purified protein with [ $^{14}C$ ]NEM (Roossien & Robillard, 1984a). Treatment of the active enzyme with NEM caused inactivation and incorporation of one label per peptide chain. This site was protected by oxygen, ferricyanide, phenylarsine oxide, and phosphorylation. Since only 1 mol of label was incorporated per peptide chain, the protection experiments suggested that an interpeptide disulfide bridge was involved. These data led to the proposal that  $EII^{Mtl}$  existed as a dimer with an interpeptide activity-linked dithiol. We have confirmed this proposal by cross-linking the peptides using the short bifunctional sulfhydryl reagents 1,3-dibromoacetone and *N,N'*-1,2-phenylenedimaleimides and by oxidizing the dithiol to a disulfide with  $Cu^{2+}$  and with  $O_2$  alone. In all cases an  $EII^{Mtl}$  dimer was observed on SDS-polyacrylamide gels (unpublished results). We have also obtained evidence for the existence of an  $EII^{Mtl}$  dimer in the membrane. When gentle extraction procedures are employed, the dimer can be solubilized in an active state, labeled by [ $^{32}P$ ]HPr, and visualized as a dimer on SDS-polyacrylamide gels. The dimer can be dephosphorylated by its substrate, mannitol (Roossien & Robillard, 1984b). Analytical ultracentrifugation on purified  $EII^{Glc}$  solubilized in octylpoly(oxyethylene) indicate that this enzyme also occurs as a dimer (Erni et al., 1982).

A different sulfhydryl group was labeled by [ $^{14}C$ ]NEM in P- $EII^{Mtl}$ . Little, if any, labeling occurred at this site in the dephosphorylated enzyme. Phenylarsine oxide prevented labeling, suggesting that this sulfhydryl could also be capable of forming an interpeptide disulfide. Reaction at this site did not cause inactivation. This site may be equivalent to the nonactivity-linked dithiol on  $EII^{Glc}$ .

**Transmembrane Channel Formed by  $EII^{Glc}$ .** The inactivation data combined with the [ $^{14}C$ ]NEM labeling studies and the cross-linking results on purified  $EII^{Mtl}$  lead to the following model of EII. An enzyme aggregate (possibly a dimer) forms a transmembrane channel. A dithiol essential for activity is located in the channel. Phosphorylation protects against alkylation presumably by effecting a conformational change that restricts access of reagents to the activity-linked site or by altering the reactivity of the dithiol. The dithiol can be oxidized by various nonpermeant oxidizing agents and, in intact cells, by a  $\Delta\mu_{H^+}$ . The oxidized enzyme is inactive both in transport and in phosphorylation.

A nonactivity-linked dithiol is located near the outer surface of the cytoplasmic membrane. Although it effects the reactivity of the activity-linked site, we have no information concerning the proximity of the two sites.

Only analytical ultracentrifugation data have been supplied in support of an  $EII^{Glc}$  dimer. Until more evidence has been accumulated, the dimer hypothesis will remain speculative. Whether the EII dimers and the channel that allows access

to the activity-linked dithiol play any role in the physical mechanism of sugar transport remain to be determined.

#### ACKNOWLEDGMENTS

We express our appreciation to P. W. Postma for supplying the *S. typhimurium* strains used in this study.

**Registry No.** PEP-sugar phosphotransferase enzyme II, 37278-09-4; dithiol, 496-74-2.

#### REFERENCES

- Abbott, R. E., & Schachter, D. (1976) *J. Biol. Chem.* **251**, 7176-7183.
- Batt, E. R., Abbott, R. E., & Schachter, D. (1976) *J. Biol. Chem.* **251**, 7184-7189.
- Begley, G. S., Hansen, D. E., Jacobsen, G. R., & Knowles, J. R. (1982) *Biochemistry* **21**, 5552-5556.
- Brouwer, M., Elferink, M., & Robillard, G. T. (1982) *Biochemistry* **21**, 82-88.
- Cohn, D. E., Kaczorowski, G. J., & Kaback, H. R. (1981) *Biochemistry* **20**, 3308-3313.
- Del Campo, F. F., Hernandez-Asensio, M., & Ramirez, J. M. (1975) *Biochem. Biophys. Res. Commun.* **63**, 1099-1105.
- Dooijewaard, G., Roossien, F. F., & Robillard, G. T. (1979) *Biochemistry* **18**, 2990-2996.
- Erni, B., Trachel, H., Postma, P. W., & Rosenbusch, J. P. (1982) *J. Biol. Chem.* **257**, 13726-13730.
- Furano, A. V. (1971) *Methods Enzymol.* **17B**, 509-511.
- Gachelin, G. (1969) *Biochem. Biophys. Res. Commun.* **34**, 382-387.
- Griffiths, D. G. (1983) Ph.D. Thesis, University of Kent.
- Griffiths, D. G., Partis, M. D., Sharp, R. N., & Beechey, R. B. (1981) *FEBS Lett.* **134**, 261-263.
- Griffiths, D. G., Partis, M. D., Moore, A. L., Proudlove, M. O., & Beechey, R. B. (1983) *Biochem. Soc. Trans.* **11**, 753-754.
- Haguenauer, R., & Kepes, A. (1972) *Biochimie* **54**, 505-512.
- Hagunauer-Tsapis, R., & Kepes, A., (1977a) *Biochim. Biophys. Acta* **469**, 211-215.
- Hagunauer-Tsapis, R., & Kepes, A. (1977b) *Biochim. Biophys. Acta* **465**, 118-130.
- Hagunauer-Tsapis, R., & Kepes, A. (1980) *J. Biol. Chem.* **255**, 5075-5081.
- Hernandez-Asensio, M., Ramirez, J. M., & Del Campo, F. F. (1975) *Arch. Microbiol.* **103**, 155-162.
- Konings, W. N., & Robillard, G. T. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3945-3948.
- Lee, C. A., & Saier, M. H., Jr. (1983) *J. Biol. Chem.* **258**, 10761-10767.
- Le Quoc, K., Le Quoc, D., & Gaudemer, Y. (1979) *Biochim. Biophys. Acta* **546**, 356-364.
- Lolkema, J. S., & Robillard, G. T. (1986) *Eur. J. Biochem.* (in press).
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 256-275.
- Misset, O., & Robillard, G. T. (1982) *Biochemistry* **21**, 3136-3142.
- Misset, O., Blaauw, M., Postma, P. W., & Robillard, G. T. (1983) *Biochemistry* **22**, 6163-6170.
- Moore, A. L., Proudlove, M. O., Portis, M. D., & Beechey, R. B. (1984) *Adv. Photosynth. Res., Proc. Int. Congr. Photosynth.*, **6th** **3**, 909-912.
- Moore, R. J., & Beechey, R. B. (1985) *Biochem. Soc. Trans.* **13**, 690-691.
- Peri, K. G., Kornberg, H. L., & Waygood, E. B. (1984) *FEBS Lett.* **170**, 55-58.
- Poolman, B., Konings, W. N., & Robillard, G. T. (1983) *Eur. J. Biochem.* **135**, 41-46.
- Reenstra, W. W., Patel, L., Rottenberg, H., & Kaback, H. R. (1980) *Biochemistry* **19**, 1-9.
- Reider, E., Wagner, E. F., & Schweiger, M. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5529-5533.
- Robillard, G. T., & Konings, W. N. (1981) *Biochemistry* **20**, 5025-5032.
- Robillard, G. T., & Konings, W. N. (1982) *Eur. J. Biochem.* **127**, 597-604.
- Robillard, G. T., Dooijewaard, G., & Lolkema, J. S. (1979) *Biochemistry* **18**, 2984-2989.
- Roossien, F. F., & Robillard, G. T. (1984a) *Biochemistry* **23**, 211-215.
- Roossien, F. F., & Robillard, G. T. (1984b) *Biochemistry* **23**, 5682-5685.
- Saier, M. H., Jr., Wentzel, D. L., Feutch, B. U., & Judice, J. J. (1975) *J. Biol. Chem.* **250**, 5089-5096.
- Scholte, B. J., Schuitema, A. R. J., & Postma, P. W. (1982) *J. Bacteriol.* **149**, 576-586.